

Characterization of the genetic locus responsible for the production of ABP-118, a novel bacteriocin produced by the probiotic bacterium *Lactobacillus salivarius* subsp. *salivarius* UCC118

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ABP-118, a small heat-stable bacteriocin produced by *Lactobacillus salivarius* subsp. *salivarius* UCC118, a strain isolated from the ileal-caecal region of the human gastrointestinal tract, was purified to homogeneity. Using reverse genetics, a DNA fragment specifying part of ABP-118 was identified on a 10769 bp chromosomal region. Analysis of this region revealed that ABP-118 was a Class IIb two-peptide bacteriocin composed of Abp118 α , which exhibited the antimicrobial activity, and Abp118 β , which enhanced the antimicrobial activity. The gene conferring strain UCC118 immunity to the action of ABP-118, *abpIM*, was identified downstream of the *abp118 β* gene. Located further downstream of *abp118 β* , several ORFs were identified whose deduced proteins resembled those of proteins involved in bacteriocin regulation and secretion. Heterologous expression of ABP-118 was achieved in *Lactobacillus plantarum*, *Lactococcus lactis* and *Bacillus cereus*. In addition, the *abp118* locus encoded an inducing peptide, AbpIP, which was shown to play a role in the regulation of ABP-118 production. This novel bacteriocin is, to the authors' knowledge, the first to be isolated from a known human probiotic bacterium and to be characterized at the genetic level.

Keywords: lactic acid bacteria, two-peptide bacteriocin, bacterial immunity

INTRODUCTION

Health-promoting bacteria, more generally referred to as probiotics, have been shown to improve the intestinal microbial balance and the properties of the indigenous microflora (Naidu *et al.*, 1999). The intestinal probiotic microflora is believed to promote health directly through its metabolic activities and its physical presence, providing benefits such as the competitive exclusion of medically significant pathogens, stimulation of the

immune system, and treatment and neutralization of the side effects of antibiotic therapy (Mattila-Sandholm *et al.*, 1999). Probiotics have been defined as living micro-organisms which upon ingestion in adequate numbers exert positive health effects beyond inherent basic nutrition (Guarner & Schaafsma, 1998). One of the desirable properties of a probiotic strain is the ability to produce antimicrobial substances such as bacteriocins (Mattila-Sandholm *et al.*, 1999).

Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptides or peptide complexes that have a bactericidal or bacteriostatic effect on other (usually closely related) species. In all cases, the producer cell exhibits genetically encoded immunity to the action of its own bacteriocin (Klaenhammer, 1993; Nes *et al.*, 1996). Based on their composition, size, heat stability, mode of action, mechanism of export and inhibitory

Abbreviations: ABC, ATP-binding cassette; AbpIP, induction factor; AU, activity unit; Bac, bacteriocin production; CFS, cell-free supernatant; LAB, lactic acid bacteria; *Lactobacillus salivarius*, *Lactobacillus salivarius* subsp. *salivarius*.

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spectrum, bacteriocins produced by lactic acid bacteria (LAB) can be categorized into three or four classes (Klaenhammer, 1993; Nes *et al.*, 1996), of which Class I and Class II are the most prevalent. Class I bacteriocins – the so-called lantibiotics – are small heat-stable peptides that owe their name to the modified amino acids with intramolecular thioether rings, such as lanthionine and β -methyl-lanthionine, present in their structures. Class II bacteriocins – the small heat-stable non-lantibiotics – are subdivided into three subcategories; IIa, pediocin-like bacteriocins with strong anti-listerial effects and a conserved N-terminal YGNGVXC consensus motif within the mature peptide; IIb, bacteriocins whose activity depends on the complementary activity of two peptides; and IIc, bacteriocins whose secretion is *sec* dependent (Klaenhammer, 1993; Nes *et al.*, 1996).

Most of the bacteriocins identified so far are synthesized as pre-peptides and contain an N-terminal leader sequence, which is removed upon externalization of the bacteriocin (Håvarstein *et al.*, 1995). The synthesis and export of these *sec*-independent bacteriocins relies upon a minimal genetic structure, generally consisting of four to five clustered genes. Such a bacteriocin gene cluster is composed of one or two structural genes (two in the case of Class IIb bacteriocins), encoding the pre-bacteriocin(s) and a specific immunity gene, which is located next to the structural gene(s). Furthermore, the cluster includes a gene encoding an ATP-binding cassette (ABC) transporter and a gene encoding an accessory protein, both of which are required for the efficient processing and externalization of the bacteriocin (except in the case of the Class IIc bacteriocins) (Nes *et al.*, 1996). The N-terminal leader sequence of the pre-bacteriocin serves as a recognition signal for the activation of the mature bacteriocin by cleavage of the leader peptide by the proteolytic domain of the transporter protein (Håvarstein *et al.*, 1994, 1995). Additional genes may be present in a bacteriocin gene cluster, such as those regulating bacteriocin production. In several cases, the regulation of bacteriocin production has been shown to be dependent on a so-called three-component regulatory mechanism, consisting of a small induction peptide (which also contains a bacteriocin-like leader peptide that may even act as a bacteriocin), a histidine protein kinase and a response regulator [for reviews see Kleerebezem *et al.* (1997) and Nes & Eijsink (1999)]. This three-component regulatory system acts as a quorum-sensing device, coupling co-ordinated bacteriocin production by a particular strain to its cell density.

In this study, we describe the purification and functional characterization of a locus involved in the production of a novel two-component bacteriocin, designated ABP-118, produced by the probiotic strain *Lactobacillus salivarius* subsp. *salivarius* (*Lactobacillus salivarius*) UCC118. We demonstrate that a 10.77 kb chromosomal region of this strain is sufficient for bacteriocin production and for conferring immunity to *Lactobacillus salivarius* UCC118 against its own bacteriocin.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Lactobacillus salivarius* UCC118 was originally isolated from the human gastrointestinal tract, as described previously (Dunne *et al.*, 1999). *Lactobacillus* strains were cultivated in MRS broth (Oxoid) at 37 °C under anaerobic (with the Merck Anaerocult A gas pak system) and aerobic conditions. *Lactobacillus* strains were also cultivated in wort obtained from the University College Cork pilot-plant brewery [the relative density of the wort was 1.061; the wort was made by using a standard mashing procedure (Analytica-EBC 4.5.1)]. Lactococcal strains were routinely propagated at 30 °C with aeration in M17 broth (Oxoid) containing 0.5% (w/v) glucose (GM17). *Escherichia coli* and *Bacillus* strains were grown at 37 °C in Luria-Bertani (LB) broth (Sambrook *et al.*, 1989) and in tryptone soya broth (Oxoid) supplemented with 0.6% (w/v) yeast extract (TSB+YE) with vigorous agitation. *Listeria* strains were grown at 37 °C in TSB+YE without agitation. The agar media were prepared by adding 1.5% (w/v) granulated agar (Difco) to the broth media; overlay agars were prepared by the addition of 0.6% (w/v) granulated agar to the broth media. Antibiotics used in the selective media were added at the following concentrations: ampicillin, 100 µg ml⁻¹; chloramphenicol, 20 µg ml⁻¹ (*Escherichia coli*), 10 µg ml⁻¹ (*Lactobacillus plantarum*, *Bacillus* and *Listeria* strains) or 5 µg ml⁻¹ (*Lactococcus lactis*); and erythromycin, 6 µg ml⁻¹ (*Lactobacillus plantarum*), 5 µg ml⁻¹ (*Lactococcus lactis* and *Listeria* strains) or 4 µg ml⁻¹ (*Bacillus* strains). LB agar supplemented with 100 mM IPTG ml⁻¹ and 40 µg X-Gal ml⁻¹ was used for the selection of transformants harbouring recombinant pGEM-T or pBluescript plasmids. The chemical reagents listed were obtained from Sigma.

Bacteriocin activity and immunity assays. The bacteriocin activity present in the neutralized (pH 6.5) cell-free supernatant (CFS) of a producing culture and the activity of purified bacteriocin were determined by critical dilution using the direct, spot-on-lawn assay (Mayr-Harting *et al.*, 1972) or the agar well-diffusion assay (Parente & Hill, 1992). Activity units (AUs) per millilitre were determined as the inverse of the last dilution at which growth inhibition was still detectable. *Bacillus coagulans* was used as the standard indicator strain, unless otherwise stated. The level of immunity of *Lactobacillus salivarius* UCC118 to ABP-118 was tested using concentrated ABP-118, obtained following ammonium sulphate precipitation (40% w/v, saturation) of the CFS from a culture of *Lactobacillus salivarius* UCC118 grown to early-stationary phase and the subsequent dialysis of the redissolved protein precipitate as described by O'Keefe *et al.* (1999).

Purification of ABP-118. ABP-118 was purified from a *Lactobacillus salivarius* UCC118 culture that had been grown in MRS broth at 37 °C until early-stationary phase (OD₆₀₀ ~0.8). The purification procedure was essentially that used for the purification of colicin V (Håvarstein *et al.*, 1994), involving ammonium sulphate precipitation followed by sequential hydrophobic interaction (Ambiclite XAD-16; Sigma) and cation-exchange chromatography (SP-Sepharose fast-flow; Pharmacia Biotech), with a final purification step by reverse-phase chromatography using a C₁₈/C₁₈ column (PeppRPC HRS/5) on an PPLC system (Pharmacia Biotech). Bacteriocin activity was eluted from the reverse-phase column with a 40 min linear gradient of 30–37% 2-propanol in aqueous 0.1% (v/v) trifluoroacetic acid (TFA), followed by a further 5 min gradient of 37–100% 2-propanol in aqueous

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Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics*	Reference/source†
Lactobacillus		
<i>L. salivarius</i> UCC118	Wild-type producer of ABP-118	Dunne <i>et al.</i> (1999)
<i>L. plantarum</i> NCIMB 8826	Isolated from human saliva	NCIMB
Lactococcus		
<i>L. lactis</i> subsp. <i>lactis</i> IL1403	Plasmid-free strain containing <i>lcnC</i> and <i>lcnD</i> analogues	Venema <i>et al.</i> (1996)
Bacillus		
<i>B. cereus</i> ATCC 14579	Plasmid-free strain sensitive to ABP-118	ATCC
<i>B. coagulans</i>	Indicator strain sensitive to ABP-118	UCC
Listeria		
<i>L. innocua</i>	Indicator strain sensitive to ABP-118	UCC
<i>L. monocytogenes</i> Scott A	Indicator strain sensitive to ABP-118	UCC
Escherichia		
<i>E. coli</i> JM109	<i>recA1 endA1 gyrA96 thi hsdR17(r_m⁻, m_m⁺) relA1 supE44 Δ(lac-proAR)</i> (F' <i>traD36 proAR lac</i> 'ZAM15)	Promega
<i>E. coli</i> XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> (F' <i>proAR lac</i> 'ZAM15 Tn10; Tet ^r)	Stratagene
Plasmids		
pGEM-T	3.0 kb PCR cloning vector; <i>alcZ</i> ; Ap ^r	Promega
pUC18	3.2 kb cloning vector; <i>alcZ</i> ; Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
pBluescript KS ⁺	3.0 kb cloning vector; <i>alcZ</i> ; Ap ^r	Stratagene
pMTL22	<i>alcZ</i> ; Ap ^r	Chambers <i>et al.</i> (1988)
pIL253	4.8 kb high-copy-number vector; Em ^r	Simon & Chopin (1988)
pNZ8048	Nisin-inducible <i>Escherichia coli</i> - <i>Lactococcus lactis</i> expression vector; Cm ^r	de Ruyter <i>et al.</i> (1996)
pNZ44	<i>Escherichia coli</i> - <i>Lactococcus lactis</i> vector containing P44 promoter; Cm ^r	McGrath <i>et al.</i> (2001)
pSF01	pGEM-T containing 80 bp PCR product corresponding to the N-terminal Abp118α peptide	This study
pSF02	pUC18 containing a 10 ⁻⁷⁷ kb <i>EcoRI</i> fragment specifying the genetic information for ABP-118 production	This study
pSF03	pBluescript containing the 10 ⁻⁷⁷ kb <i>abp118</i> locus	This study
pSF05	pNZ8048 containing 10 ⁻⁷⁷ kb <i>abp118</i> locus	This study
pSF07	pNZ8048 containing the <i>abp118</i> locus with <i>PvuII</i> deletion in <i>abp118α</i>	This study
pSFM1	pNZ44 <i>abp118α abp118β abpIM</i>	This study
pSFM2	pNZ44 <i>abp118β abpIM</i>	This study
pSFM3	pNZ44 <i>abpIM</i>	This study
pSFM4	pNZ44 <i>abp118α abpIM</i>	This study
pSFM16	pIL253 containing constitutive P27 promoter from <i>Lactococcus lactis</i> phage Tue2009 upstream of <i>abpT</i> and <i>abpD</i>	This study

* Ap^r, ampicillin resistance; Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance.

† NCIMB, National Culture of Industrial and Marine Bacteria, Aberdeen, UK; ATCC, American Type Culture Collection, Manassas, VA, USA; UCC, University College Cork strain collection.

0.1% TFA (flow rate of 0.5 ml min⁻¹). Fractions exhibiting high bacteriocin activity were freeze-dried, dissolved in aqueous 0.1% TFA and re-run on the reverse-phase column to remove any residual impurities. The critical dilution method described above was used to quantify the bacteriocin activity at the various steps of the purification process.

Amino acid sequence analysis and MS. Purified ABP-118 was hydrolysed and analysed on an amino acid analyser, as described by Fykse *et al.* (1988). The amino acid sequence was determined by Edman degradation with an Applied Biosystems model 477A automatic sequencer equipped with an

on-line 120A phenylthiohydantoin amino acid analyser (Cornwell *et al.*, 1988). MS was performed with a Biosystems Voyager-DE STR Biospectrometry Workstation.

DNA isolation. Plasmid DNA was isolated from *Escherichia coli* strains using the QIAprep Spin Miniprep Kit (Qiagen) and was isolated from *Lactococcus lactis* strains using the method of Anderson & McKay (1983). Plasmid DNA from *Lactobacillus* transformants was isolated by the method of Birnboim & Doly (1979). *Bacillus* and *Listeria* plasmid DNA was isolated using the QIAprep Spin Miniprep Kit, following treatment of a 10 ml culture of mid-exponential growing cells

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with protoplast buffer (20 mM Tris/HCl; 5 mM EDTA; 7.5 mM sucrose; 10 mg lysozyme ml⁻¹; 50 U mutanolysin ml⁻¹) at 37 °C for 30 min to effect cell lysis. Total genomic DNA of *Lactobacillus salivarius* UCC118 was isolated according to the method of Pospiech & Neumann (1995) with some modifications (outlined below). *Lactobacillus salivarius* UCC118 was grown in 100 ml MRS supplemented with 40 mM D-threonine to an OD₆₀₀ of ~0.6 and harvested by centrifugation. The pelleted cells were stored overnight at -20 °C. Cells were lysed using lysozyme (8 mg ml⁻¹) in combination with mutanolysin (50 U ml⁻¹) on ice for 45 min, followed by incubation at 37 °C for 30 min. Before adding SDS solution, 2 mg proteinase K ml⁻¹ was added to the lysate and it was incubated at 55 °C for a further 45 min. The DNA was further purified by CsCl density-gradient centrifugation (Sambrook *et al.*, 1989).

DNA manipulations. *Escherichia coli*, *Lactococcus lactis*, *Lactobacillus*, *Listeria* and *Bacillus* strains were transformed by electroporation by using the methods of Sambrook *et al.* (1989), Holo & Nes (1989), Aukurst & Blom (1992), Park & Stewart (1990) or Schurter *et al.* (1989), respectively. All electroporations were carried out with the use of a Gene-Pulser apparatus (Bio-Rad). Restriction enzymes, RNase-free DNase, T4 DNA ligase and Shrimp alkaline phosphatase were used as directed by the manufacturer (Roche Diagnostics). PCR amplification was performed by standard procedures, with BIOTAQ DNA polymerase (Bioline UK) or the Expand High Fidelity PCR System (Roche Diagnostics) and by the use of an Omnigene thermocycler (Hybaid). Thirty cycles of PCR were performed with the following conditions: 1 min at 94 °C, 1 min at an annealing temperature which ranged from 45 °C to 55 °C and an elongation step of 1–3 min at 72 °C. Oligonucleotide primers for PCR and sequencing were synthesized using an Oligo 1000M DNA Synthesizer (Beckman Instruments). DNA fragments were isolated and purified from agarose gels using the GeneClean II Kit (BIO 101). PCR products were purified using the High Pure PCR Purification Kit (Roche Diagnostics) and cloned into the plasmid vector pGEM-T (Promega). The PCR DIG Probe Synthesis Kit (Roche Diagnostics) was used to generate a non-radioactive hybridization probe complementary to the sequence specifying the N-terminal 26 aa sequence of the ABP-118 structural gene. Southern- and colony-blot hybridizations onto Hybond-N⁺ nylon membranes (Amersham) were performed by standard methods (Sambrook *et al.*, 1989); positive signals were detected by using the DIG Detection Kit (Roche Diagnostics).

DNA sequencing and analysis. DNA sequencing was performed using a 373A automated DNA sequencer (Applied Biosystems) or the CEQ 2000 Dye Terminator Cycle Sequencing Kit (Beckman Coulter), using synthetic oligonucleotides in a primer-walking strategy. Antisense primers were used to confirm the sequence on the complementary strand of DNA. Analyses of the DNA and protein sequences were performed using the DNASTAR software package. Database searches were performed using the BLAST program (Altschul *et al.*, 1997), with the latest release of the non-redundant databases of the NCBI (<http://www.ncbi.nlm.nih.gov>). The sequence presented here has been submitted to GenBank, where it has been assigned the accession number AF408405.

Plasmid construction and mutational analysis. A number of strategies were used to clone and analyse the genes involved in ABP-118 production and immunity. Plasmid pSF01 contains a DNA fragment corresponding to the N-terminal sequence of the mature Abp118 peptide cloned into pGEM-T (Promega). Plasmid pSF03 was constructed by cloning a 10.77 kb chromosomal *EcoRI* fragment, which contains the genetic information

for ABP-118 production, into the *EcoRI* site of pBluescript. The 10.77 kb fragment was excised from between the *BamHI* and *XhoI* sites of pBluescript and inserted into the *BamHI* and *XhoI* sites of pNZ8048, to obtain plasmid pSF05. The restriction of pSF05 with *PvuII* (the two *PvuII* sites of pSF05 are located within the *abp118x* gene) and the subsequent self-ligation of the resulting largest DNA fragment allowed the construction of pSF07, containing an in-frame deletion of 72 bp in the *abp118x* gene. The integrity of this deletion was confirmed by sequence analysis. To investigate the role of *abp118x*, *abp118y* and *abp118z* in ABP-118 production and immunity, pSFM1, pSFM2 and pSFM3 (Table 1) were generated by using high-fidelity PCR and primers which introduced restriction sites to facilitate cloning downstream of the constitutive lactococcal promoter in the expression vector pNZ44. The plasmid pSFM1 contains the two proposed structural genes for ABP-118 activity upstream of the proposed immunity gene, whereas pSFM2 specifies only one of the proposed structural genes, *abp118y*, upstream of the proposed immunity gene. The pNZ44 derivative, pSFM3, contains the proposed ABP-118 immunity gene, *abp118i*, on its own. The SOEing technique (Horton *et al.*, 1990) was used to generate a PCR product encompassing the *abp118x* structural gene immediately downstream of the *abp118i* gene, which was subsequently cloned into pNZ44 to generate pSFM4. A PCR fragment corresponding to *abpT* and *abpD*, the proposed genes responsible for ABP-118 transport, was cloned into the *NotI* and *EcoRI* sites of the *Escherichia coli* vector pMTL22 (Chambers *et al.*, 1988), directly downstream of the constitutive lactococcal P27 promoter from the *Lactococcus lactis* phage Tuc2009 (D. van Sinderen, unpublished results), which was amplified by PCR using primers containing the restriction sites *BglII* and *NcoI*. This construct was restricted with *BglII* and *EcoRI*; the resulting excised fragment, containing the ABP-118 transport operon cloned in the proper orientation downstream of the constitutive promoter, was subcloned between the *BamHI* and *EcoRI* sites of pIL253, generating pSFM76.

Induction factor synthesis and induction assays. The ABP-118 induction factor (AbpIP) was synthesized at MWG-Biotech and purified to >95% homogeneity by reverse-phase HPLC with an acetonitrile/H₂O gradient. The molecular mass was verified by matrix assisted laser desorption/ionization time of flight (MALDI-TOF) MS (Voyager-DE; R.P. Perspective Biosystems). To assay for biological activity, a 10⁻⁷ M stock solution of AbpIP was prepared in sterile distilled water. To lose bacteriocin activity (Bac⁻), a fully grown culture of *Lactobacillus salivarius* UCC118 was inoculated (1%) into wort and allowed to grow anaerobically at 37 °C until it was turbid. The CFS was assayed for loss of bacteriocin activity against the indicators *Listeria innocua* and *Bacillus coagulans*. The resulting Bac⁻ culture was inoculated (1%) into wort containing AbpIP at concentrations of 10⁻¹–10⁻¹⁴ M and incubated at 37 °C for 18 h. It was then tested for bacteriocin production.

RESULTS

Purification of ABP-118

The purification strategy and results obtained for ABP-118 are summarized in Table 2. The final purification step was repeated twice to obtain a pure sample. Passage through a cation-exchange column prior to FPLC decreased the specific activity (Table 2, Fraction III) but resulted in better purification. Upon application of

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Table 2. Purification of ABP-118

All results are representative of three replications.

Purification stage (fraction)	Vol. (ml)	Total A_{250}^a	Total activity (AU)	Specific activity†	Increase in specific activity (fold)	Yield (%)
Culture supernatant	2000	30780	6.4×10^8	2.08×10^3	1	100
Ammonium sulphate precipitate (I)	100	802	5.1×10^6	6.38×10^3	31	80
Hydrophobic interaction (II)	20	31	2.1×10^8	1.31×10^4	629	32
Cation exchange (III)	20	6.8	5.1×10^6	7.53×10^4	362	8
C_3/C_{15} reverse-phase FPLC (IV)‡	1	0.26	4.1×10^6	1.59×10^5	7644	6.4

^a $A_{250} \times \text{vol. (ml)}$.† Total activity (AU) divided by the total A_{250} .

‡ Second FPLC step.

Fraction III to the reverse-phase column, ABP-118 activity eluted as a single absorbency peak at 214 nm, corresponding to a concentration of about 100% (v/v) 2-propanol (data not shown). The purified bacteriocin was found to have the same inhibitory spectrum as the supernatant of the culture (data not shown).

Amino acid sequence analysis and MS

The N-terminal sequence of the first 29 aa of the peptide exhibiting inhibitory activity was determined by Edman degradation and generated the following sequence: KRGPNXVGNFLGGLFAGAAAGVPLGPAGI (where X is an undetermined amino acid). Amino acid compositional analysis did not reveal the presence of unusual residues (data not shown) commonly found in the lantibiotics, such as lanthionine, β -lanthionine or dehydrated residues. The molecular mass of the purified bacteriocin activity, as determined by MS, was 4096.69 Da, indicating that the bacteriocin activity corresponded to a single peptide composed of 45 or 46 aa.

Identification and cloning of the genetic determinants for ABP-118 production

Using the degenerate primers 3137 (5'-GGNAAACGN-GGNCCNAAC-3') and 3139 (5'-GGGCCCT(A/G)NG-GNACNCC-3'), a 78 bp PCR fragment, corresponding to the DNA region encoding the 26 N-terminal amino acids of the mature ABP-118 protein, was amplified from the genomic DNA of *Lactobacillus salivarius* UCC118, sequenced to confirm its expected sequence and then cloned into pGEM-T, generating pSF01. Total or plasmid DNA preparations isolated from *Lactobacillus salivarius* UCC118 were digested with a range of restriction enzymes, transferred to a nylon membrane and used in a Southern hybridization experiment employing the insert of pSF01 as a probe. Hybridization signals were observed in specific fragments obtained from total DNA digests, whereas plasmid DNA did not

generate hybridizing signals, indicating that the ABP-118 structural gene is chromosomally encoded (data not shown). In particular, hybridization of the probe was observed to an approximately 10.77 kb *EcoRI* chromosomal DNA fragment. Shotgun cloning of an *EcoRI* restriction digest of genomic DNA into the *EcoRI* site of pUC18 was performed. Two *Escherichia coli* transformants were shown, by colony hybridization, to harbour a recombinant plasmid containing the 10.77 kb *EcoRI* fragment; one of these, designated pSF02, was retained for sequence analysis.

Heterologous expression of ABP-118

The 10.77 kb bacteriocin-encoding chromosomal fragment of *Lactobacillus salivarius* UCC118 was cloned into the high-copy-number vector pNZ8048 to generate pSF05, using *Escherichia coli* as the intermediate host. The resulting plasmid was introduced into a number of bacterial species which were then tested for their ability to produce ABP-118. Plasmid pSF05 conferred *Lactobacillus plantarum* NCIMB 8826, *Lactococcus lactis* IL1403 and *Bacillus cereus* ATCC 14579 with the ability to produce ABP-118 (Fig. 1). Overnight cultures of the transformants produced lower levels of bacteriocin than wild-type *Lactobacillus salivarius* UCC118. In addition, when pSF07, a derivative of pSF05 encoding the entire ABP-118 operon but carrying an in-frame deletion in the *abp118a* structural gene (see below), was introduced into *Lactobacillus plantarum* NCIMB 8826 and *Lactococcus lactis* IL1403 the resulting transformants failed to produce bacteriocin (data not shown). Thus, deletion of one of the ABP-118 structural genes results in the loss of bacteriocin production.

Sequence analysis of the *abp118* gene cluster

The complete nucleotide sequence of the 10.77 kb *EcoRI* fragment was determined by a primer-walking strategy, the analysis of which showed that the fragment consisted of 10769 bp and revealed the presence of 16 complete

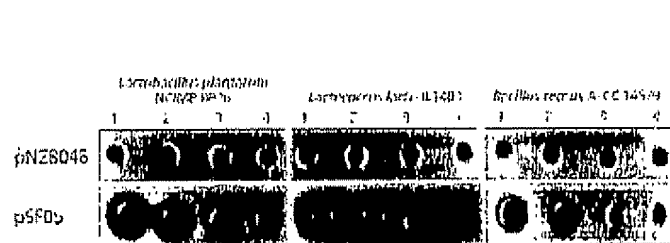


Fig. 1. Inhibitory activity of a twofold serial dilution of ABP-118 secreted by *Lactobacillus plantarum* NCIMB 8826, *Lactococcus lactis* IL1403 or *Bacillus cereus* ATCC 14579, containing either the control plasmid pNZ8048 (upper panel) or pSF85 (lower panel). *Bacillus coagulans* was used as the indicator strain. Numbers above the wells correspond to the CFS dilution in each well. 1, Undiluted CFS; 2, 1:2 dilution of CFS; 3, 1:4 dilution of CFS; 4, 1:8 dilution of CFS.

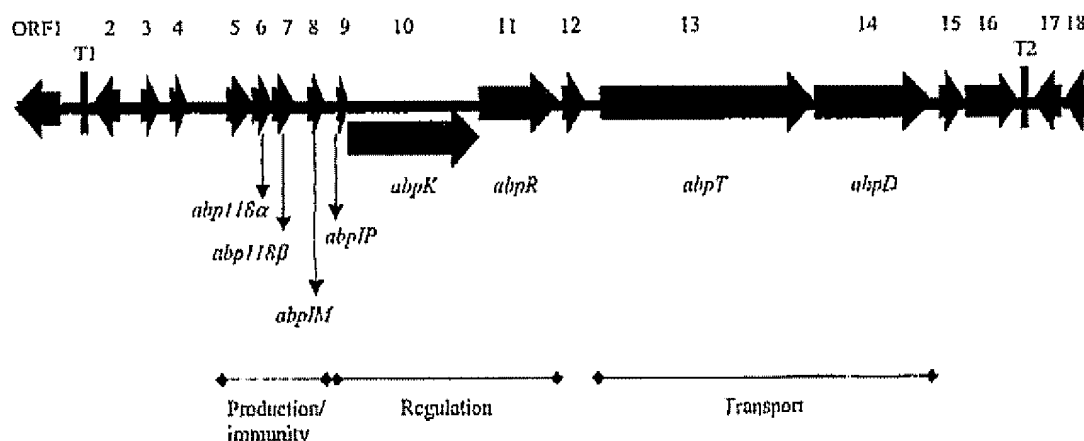


Fig. 2. Graphic illustration of the *abp118* locus showing 18 potential ORFs (indicated by arrows). ORFs with a deduced function in the production and secretion of active ABP-118 are denoted by the prefix *abp* (see Results for explanation). The presence of putative transcriptional terminators (T1 and T2) is indicated by vertical solid bars. Sections of the *abp118* locus assumed to be dedicated to production/immunity, regulation and transport are indicated.

putative ORFs and two incomplete ORFs (Fig. 2; ORFs were numbered 1–18, but some were re-named if a function could be assigned to their encoded products). The ORFs were identified based on the adopted criteria that an ORF consists of at least 35 codons preceded by a potential Shine–Dalgarno sequence at an appropriate distance (6–15 bp) from one of the commonly used initiation codons (AUG, UUG, GUG) (Harley & Reynolds, 1987). ORFs 3–16 were all oriented in the same direction. ORF1 and ORF2 (located upstream of ORFs 3–16) and ORF17 and ORF18 (located downstream of ORFs 3–16) were oriented in the opposite direction (Fig. 2). ORF4 appeared to employ a GUG translation initiation codon, whereas ORF11 (*abpR*), ORF13 (*abpT*), ORF15 and ORF16 were assumed to initiate translation from a UUG start codon. All of the remaining ORFs appeared to use an AUG initiation codon. A pair of short direct repeats with a single mismatch were located in the non-coding region between ORF4 and ORF5 [CTTATAGGG(12nt)CTTCTAGGG]. Inverted repeats typical of *rho*-independent transcription-termination signals (Tinoco *et al.*, 1973) were found one nucleotide downstream of ORF2 (T1) and 35 bp downstream of ORF16 (T2), respectively (Fig. 2).

Analysis of the gene products encoded by the *abp118* locus

The primary structures of the deduced products of ORFs 3, 4, 5, 6, 7 and 9 (Table 3) all resembled Class II bacteriocin precursors, which are typically small, cationic, bacteriocin-like peptides containing double-glycine-type leaders and having relatively high pI values (Håvarstein *et al.*, 1994; Nes *et al.*, 1996). The leaders of ORFs 3, 6, 7 and 9 conformed closely to the consensus leader sequence proposed by Nes *et al.* (1996), having hydrophobic residues present at positions –4, –7, –12 and –15 and hydrophilic residues at positions –8, –10 and –11. ORF4 and ORF5 only possessed the consensus amino acids at positions –4, –10, –11 and –12 (Table 3).

Based on the N-terminal-amino-acid sequencing results, the gene encoding ABP-118 activity, designated *abp118α*, corresponded to ORF6 (Fig. 2). No noticeable difference was observed between the calculated and experimentally determined molecular mass of the mature *Abp118α* product, indicating the absence of any major post-translational modifications. Both *abp118α* and ORF7 (designated *abp118β*), located directly downstream of *abp118α*, exhibited significant sequence similarity to

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Table 3. Pre-peptides of the *abp118* locus containing double-glycine leader sequences

Protein	Amino acid sequence of pre-peptide ^a	Pre-peptide		Mature peptide		
		Size (aa)	pI Value	Size (aa)	pI Value	
ORF3	ANNNRQVDSKSLAHKCG	ANSYVYNSGQPGCTD	57	8.06	32	6.00
ORF4	VLRKLVNFWLDGG	LIRGRKRYVIRNFARFLSNWLSNDEGMAYLYY	50	9.37	37	9.70
ORF5	NLIKLETFWLDGG	FIRGKRYVIRNFARFLSNWLSNDEGMAYLYY LISYASGVNKHMRG	85	9.37	72	9.47
ORF6 (Abp118α)	ANKKEFTVLTCELAHVGG	KRPNCVGNLGGFLPAGAAAGVLPAGIVGGANLGAIVGGALTCI	64	6.32	45	6.63
ORF7 (Abp118β)	ANKNLRKFTMTEDNLASYNGG	KNGYGSQNTKVVHCGAGIVGGALGAAGVSAVAGGSGFTSCN	60	9.13	46	9.31
ORF9 (Abp118γ)	ANKKEFTVLTCELAHVGG	ATIKGGPKRWQCFPTFGVCK	36	10.01	21	10.01
Consensus†	-x-D-Ox-x-GG					

^a x, Hydrophobic residue; O, hydrophilic residue. The arrow indicates the conserved cleavage site.

† Suggested consensus sequence (Nes *et al.*, 1996).

Table 4. Similarities observed for the proteins specified by the identified ORFs of the *abp118* gene cluster to their homologues

Only the best matches are shown. The P-values were derived from the TBLASTP score and represent the probability that the observed similarity had occurred by chance. Low P-values indicate a high probability of two proteins being structurally related.

ORF (gene)	Size (aa)	Homologue			
		Comment	P-value	Match (%) ^a	Reference
ORF1	150	Accessory transport protein, brochoxin-C system	0.30	12 (158)	McCormick <i>et al.</i> (1998)
ORF2	87	No homologues			
ORF3	57	Presalivarin B of <i>Lactobacillus salivarius</i> M6	8×10^{-23}	98 (57)	CAB63109†
ORF4	50	No homologues			
ORF5	85	No homologues			
ORF6 (<i>abp118α</i>)	64	BlpJ, auto-induced peptide of <i>Streptococcus pneumoniae</i>	1×10^{-4}	29 (89)	de Saizieu <i>et al.</i> (2000)
ORF7 (<i>abp118β</i>)	68	Plantaricin 1.25β of <i>Lactobacillus plantarum</i>	8×10^{-2}	27 (69)	Fuhrmann <i>et al.</i> (2000)
ORF8 (<i>abp118γ</i>)	55	No homologues			
ORF9 (<i>abp118δ</i>)	38	Competence pheromone of <i>Streptococcus gordonii</i>	2.2	20 (50)	Hävarstein <i>et al.</i> (1996)
ORF10 (<i>abpK</i>)	429	Histidine kinase, carnobacteriocin A system	1×10^{-40}	32 (425)	Franz <i>et al.</i> (2000)
ORF11 (<i>abpR</i>)	264	Response regulator, carnobacteriocin A system	5×10^{-30}	38 (254)	Franz <i>et al.</i> (2000)
ORF12	79	No homologues			
ORF13 (<i>abpI</i>)	719	ABC transporter, plantaricin A system	0.0	69 (716)	Diep <i>et al.</i> (1994)
ORF14 (<i>abpD</i>)	384	Accessory transport protein, sakacin A system	1×10^{-43}	40 (461)	Axelsson & Holck (1995)
ORF15	76	No homologues			
ORF16	174	No homologues			
ORF17	88	No homologues			
ORF18	80	No homologues			

^a The number of amino acids over which the percentage match was determined is shown in parentheses.

† Accession number of sequence submitted directly to EMBL Database.

each other as well as to a number of putative Class II pre-bacteriocin peptides (Table 4). Some of these pre-bacteriocin peptides correspond to one member of a Class IIb two-component bacteriocin. This similarity, which was particularly pronounced in the N-terminal region of these proteins, was due mainly to the conserved residues in the leader sequence, common to most Class II pre-bacteriocins, whereas the mature peptides showed limited similarity to any previously described bacterio-

cin. *Abp118β* was found to constitute the second peptide of a two-component bacteriocin (see below). *Abp118α* and *Abp118β* had a high proportion of glycine residues (28.9 and 32.6 %, respectively) spread evenly along their entire length; this appears to be a common feature of Class II bacteriocins (Klaenhammer, 1993), probably allowing these peptides a high degree of conformational freedom (Ennehar *et al.*, 2000). ORF8, designated *abp118M* and encoding immunity to the antimicrobial

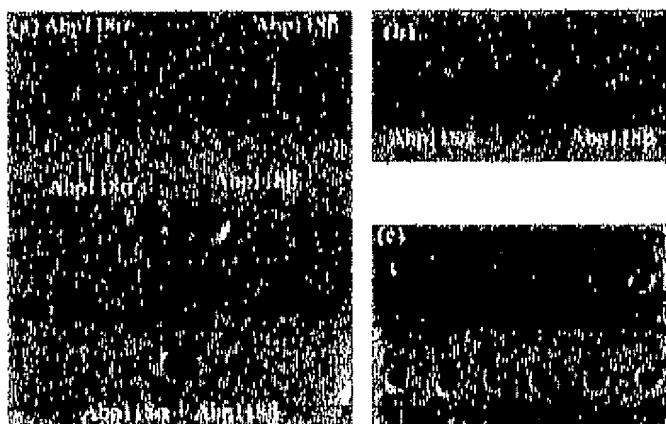


Fig. 3. (a) Bacteriocin activity and extra-cellular complementation of the Abp118 α and Abp118 β peptides. CFS (25 μ l) of *Lactobacillus plantarum* NCIMB 8826 containing pSFM4 (Abp118 α) and CFS (25 μ l) *Lactobacillus plantarum* NCIMB 8826 containing pSFM2 (Abp118 β) was added to the wells in plates seeded with the ABP-118-sensitive indicator *Listeria innocua*. CFS (25 μ l) from each culture was mixed and then added to the well labelled Abp118 α +Abp118 β . (b) 'Concentrated CFS' (25 μ l) of *Lactobacillus plantarum* NCIMB 8826 containing pSFM4 (Abp118 α) and 'concentrated CFS' (25 μ l) of *Lactobacillus plantarum* NCIMB 8826 containing pSFM2 (Abp118 β) were added to adjacent wells in plates seeded with the ABP-118-sensitive indicator *Listeria innocua*. (c) Sensitivity of *Listeria monocytogenes* ScottA containing pNZ44 (upper panel) and *Listeria monocytogenes* ScottA containing pSFM3 (lower panel) to a twofold serial dilution of concentrated ABP-118 (51600 AU ml⁻¹).

activities of the *Lactobacillus salivarius* UCC118 culture, was located immediately downstream of *abp118 α* and *abp118 β* (see below). The deduced protein of ORF3 was nearly identical to the antibacterial peptide precursor presalivaricin B (accession no. CAB63109) (Table 4), differing by only one amino acid at residue 42. The deduced product of ORF4 was very similar over its entire length to the peptide product of ORF5, which extended for another 35 aa (Table 3).

The proteins encoded by ORF9 (designated *abpI*), ORF10 (designated *abpK*) and ORF11 (designated *abpR*) exhibited significant sequence similarity to a small, cationic competence pheromone with a double-glycine-type leader, to histidine protein kinase and to response regulator proteins, respectively (Table 4). These three associated genes therefore seemed to represent a so-called three-component regulatory system, similar to those involved in the control of several Class II bacteriocins (Kleerebezem *et al.*, 1997; Nes & Eijsink, 1999; see below).

The protein products of the adjacent downstream positioned genes ORF13 and ORF14 exhibited high similarity to ABC-transporter and accessory proteins, and these genes were designated as *abpT* and *abpD*, respectively (Table 4). It is well established that the secretion of bacteriocin peptides possessing a double-glycine-type leader is mediated by a dedicated trans-membrane translocator belonging to the HlyB ABC-transporter superfamily and an accessory protein (H  varstein *et al.*, 1995; Nes *et al.*, 1996).

ABP-118 is composed of two peptides

To establish whether both *abp118 α* and *abp118 β* were essential for bacteriocin ABP-118 production, *abp118 α* and *abp118 β* were cloned both separately and in

combination upstream of an intact *abpIM* immunity gene (see below), under the control of a constitutive lactococcal promoter in the expression vector pNZ44. The resulting transformants were called pSFM1, pSFM2 and pSFM4 (Table 1). Subsequently, pSFM1 was introduced into *Lactobacillus plantarum* NCIMB 8826 with and without the construct pSFM6, encoding the putative ABP-118 transport and processing genes *abpT* and *abpD*, which were also under the control of a constitutive lactococcal promoter. Only *Lactobacillus plantarum* NCIMB 8826 transformants containing both pSFM1 and pSFM6 were capable of producing antimicrobial activity. The inhibition observed against the indicator strain was eliminated when the CFSs of these transformants were treated with 50 μ g proteinase K ml⁻¹, confirming the expected proteinaceous nature of the inhibitory compound. This implied that *abpT* and *abpD* were necessary for the production of active ABP-118 in the CFS of a heterologous strain, and it was assumed that these genes encode the dedicated transport and processing functions.

Lactobacillus plantarum NCIMB 8826 clones containing either pSFM2 or pSFM4 in conjunction with pSFM6 were assayed for inhibitory activity by the agar well-diffusion assay against the indicator *Listeria innocua* (Fig. 3). No zone of inhibition was observed surrounding the wells containing the CFSs of either the pSFM2 or the pSFM4 transformants. However, when the CFSs of each transformant type were placed in wells adjacent to each other, a zone of enhanced inhibition was observed between the wells (Fig. 3a). The inhibitory activity of the CFSs of both transformant types was concentrated 200-fold by ammonium sulphate precipitation, resuspension and dialysis (see Methods). When the 'concentrated CFSs' from both transformant types were assayed for inhibitory activity, it was found that pSFM4 transfor-

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nants possessed antimicrobial activity, whereas none was associated with the concentrated CFS of pSFM2 transformants (Fig. 3b). The strongest zone of inhibition was observed between the wells, indicating that the concentrated CFS of pSFM2 transformants retained its ability to enhance the intrinsic inhibitory activity associated with the concentrated CFS of pSFM4 transformants (Fig. 3b). Enhancement of the antibacterial activity in the CFS of transformants containing either pSFM2 or pSFM4 with the CFS of transformants containing pNZ44 as a control was not observed (data not shown).

ORF8 represents the gene involved in conferring immunity to ABP-118

The assumption that ORF8, designated here as *abpIM* and located downstream of *abpI18*, encoded immunity was largely based on the fact that in many bacteriocin-encoding gene clusters the immunity gene is located immediately downstream of the structural gene(s), and that such immunity-conferring proteins are hydrophobic (Nes *et al.*, 1996). An expression clone, pSFM3, containing only *abpIM* was constructed to confirm the role of this gene in immunity to ABP-118. Plasmid pSFM3 was introduced into *Listeria monocytogenes* ScottA, which is sensitive to ABP-118, and this strain was subsequently used as an indicator. *Listeria* transformants of pSFM3 were immune to 21600 AU ABP-118 ml⁻¹, although they were not completely immune to ABP-118 at the highest concentration (51200 AU ABP-118 ml⁻¹) (Fig. 3c). Thus, the insert on pSFM3 is capable of conferring ABP-118 immunity to a previously sensitive strain.

Bacteriocin production is inducible in *Lactobacillus salivarius* UCC118

Spontaneous loss of bacteriocin production was not observed when standard cultures of *Lactobacillus salivarius* UCC118 were diluted 10⁻⁶ times or more in MRS medium, as has been observed for many three-component regulated Class II bacteriocin-producing bacteria (Nes & Eijsink, 1999). However, production of ABP-118 was abolished when *Lactobacillus salivarius* UCC118 was grown in wort. Once *Lactobacillus salivarius* UCC118 had lost the ability to produce bacteriocin (Bac⁻), subcultures from it continued to be Bac⁻ when grown in wort. The addition of sterile CFS from a Bac⁺ culture of *Lactobacillus salivarius* UCC118 induced bacteriocin production, whereas CFS derived from a Bac⁻ culture did not. The inducing component was lost after treatment of the CFS with proteinase K, indicating that the culture contained a protein component that was indispensable for maintaining bacteriocin production. To determine whether the product of *abpIP* was the induction factor for ABP-118 production, the chemically synthesized mature peptide (AbpIP) was used to induce bacteriocin production in a Bac⁻ culture of *Lactobacillus salivarius* UCC118 grown in wort. Addition of AbpIP at concentrations above 10⁻¹⁰ M restored bacteriocin pro-

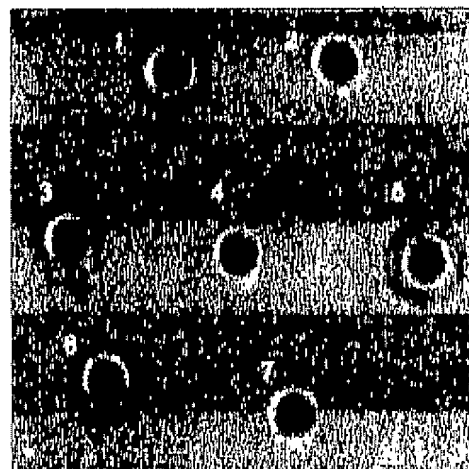


Fig. 4. Inhibitory activity of ABP-118 against the indicator *Bacillus coagulans*. Inhibitory activity of ABP-118 in: 1, the CFS of wild-type *Lactobacillus salivarius* UCC118; 2, a Bac⁻ derivative of *Lactobacillus salivarius* UCC118 subcultured in wort; 3, a Bac⁻ derivative of *Lactobacillus salivarius* UCC118 subcultured in wort containing 20 µl of CFS from a Bac⁺ culture of *Lactobacillus salivarius* UCC118; 4, a Bac⁻ derivative of *Lactobacillus salivarius* UCC118 subcultured in wort containing 20 µl of CFS from a Bac⁺ culture of *Lactobacillus salivarius* UCC118 treated with proteinase K; 5, a Bac⁻ derivative of *Lactobacillus salivarius* UCC118 subcultured in MRS; 6, a Bac⁻ derivative of *Lactobacillus salivarius* UCC118 subcultured in wort containing 10⁻⁶ M AbpIP; and 7, a Bac⁻ derivative of *Lactobacillus salivarius* UCC118 subcultured in wort containing 10⁻⁶ M AbpIP treated with proteinase K.

duction (Fig. 4). When inoculated into wort without AbpIP, the Bac⁻ culture failed to produce bacteriocin and served as a negative control. Proteinase K-treated AbpIP did not induce bacteriocin production, demonstrating that the observed induction was AbpIP specific (Fig. 4). In all cases, whether bacteriocin production was switched on or off following growth in wort, subsequent subculturing into MRS broth resulted in bacteriocin-producing cultures.

DISCUSSION

This paper describes the identification and genetic analysis of a novel chromosomal locus responsible for the production of a two-component bacteriocin, ABP-118, from the probiotic bacterium *Lactobacillus salivarius* UCC118, which was originally isolated from a human intestinal tract (Dunne *et al.*, 1999). To our knowledge, this is the first published report on the genetic characterization of a bacteriocin locus from a human-derived probiotic strain. ABP-118 is an intriguing broad-spectrum antimicrobial, since it is capable of inhibiting a number of food-borne and medically significant pathogens, including *Bacillus*, *Listeria*, *Enterococcus* and *Staphylococcus* species, without an apparent antagonistic activity towards related LAB

strains, with the exception of *Lactobacillus fermentum* KLD (Dunne *et al.*, 1999). In many cases, bacteriocins produced by LAB inhibit species that are closely related to the producing strain (Marciset *et al.*, 1997).

Our results indicate that the antimicrobial activity arises from the complementary action of two closely related, small, unmodified cationic and hydrophobic peptides, termed Abp118 α and Abp118 β . The Abp118 α peptide possesses inhibitory activity when concentrated, and this activity is enhanced by the presence of the Abp118 β peptide, which on its own has no associated bacteriocin activity. This makes ABP-118 a member of the Class IIb bacteriocins. In some two-peptide systems, including lactococcin G and brochoacin-C, both peptides are needed for activity (Nissen-Meyer *et al.*, 1992; McCormick *et al.*, 1998). In others, such as lactacin F and thermophilin 13, one or both peptides exhibit low but measurable inhibitory activity on their own, but the combined action of both peptides enhances the activity much more than the additive effect of the peptides acting independently (Allison *et al.*, 1994; Marciset *et al.*, 1997).

The genes responsible for ABP-118 production were located on a chromosomally derived 10.77 kb DNA fragment, and this locus was sufficient to support the heterologous expression of active antimicrobial activity. The organization of the (putative) genes required for ABP-118 production, secretion and regulation is similar to that of other Class II bacteriocin gene clusters (Nes *et al.*, 1996). The adjacent genetic elements *abp118 α* , *abp118 β* and *abp1M* were identified as the structural gene, the enhancer gene and the putative immunity gene, respectively, for ABP-118. When expressed in *Listeria monocytogenes* ScottA, *abp1M* conferred only partial immunity to the highest bacteriocin level used and this partial immune phenotype may be due to lower levels of transcription of *abp1M* under the control of a lactococcal promoter in this heterologous host.

ABP-118 production appears to be controlled by a so-called three-component quorum-sensing regulatory system consisting of AbpIP, AbpK and AbpR (Brurberg *et al.*, 1997; Diep *et al.*, 2000; Kleerebezem *et al.*, 1997; Nes & Eijsink, 1999). In a situation similar to that observed for sakacin A production by *Lactobacillus sakei* LTH673, *Lactobacillus salivarius* UCC118 does not lose the Bac⁻ phenotype upon extreme dilution of the culture, a strategy used successfully for obtaining a Bac⁻ phenotype for other bacteriocin systems (Diep *et al.*, 2000). Down-regulation of bacteriocin production is known to occur under different environmental conditions. Changes in growth media, temperature and pH can induce spontaneous loss of bacteriocin production (Brurberg *et al.*, 1997; Diep *et al.*, 2000). Growth of *Lactobacillus salivarius* UCC118 in wort resulted in a Bac⁻ phenotype; reversion to a Bac⁺ phenotype was observed only when wort was supplemented with chemically synthesized AbpIP or with CFS. Analogous to other systems, it is likely that bacteriocin production in *Lactobacillus salivarius* UCC118 is controlled by an auto-induction mechanism involving the secreted pep-

tide pheromone AbpIP. Further investigations are required to determine the nature of the constituent(s) in wort which affect regulation of bacteriocin production.

Lactobacillus salivarius UCC118 appears to have the genetic potential on the 10.77 kb *EcoRI* fragment to produce several other Class II bacteriocin-like peptides. It is not uncommon that identical bacteriocins are produced by different strains of the same species, or by different bacterial species belonging to the same genus (Ennahar *et al.*, 2000). ORF3 specifies a peptide that is almost identical to presalivaricin B, a bacteriocin produced by *Lactobacillus salivarius* M6 (ten Brink *et al.*, 1994). An internal deletion in the *abp118 α* gene abolished all bacteriocin production in the heterologous hosts *Lactobacillus plantarum* and *Lactococcus lactis* IL1403. This leaves the possibility that the presalivaricin-B-encoding gene is not expressed, is not processed and/or secreted, or that the single amino acid difference could have rendered the ORF3-specified peptide inactive. Similar single amino acid changes have been shown to have a profound effect on the biological activity of carnobacteriocin B2 and mesentericin Y (Fleury *et al.*, 1996; Quadri *et al.*, 1997).

To our knowledge, this is the first published report of a bacteriocin isolated from a probiotic strain of human origin to be purified and genetically analysed. Although the health-contributing effects of a bacteriocin-producing bacterium may be difficult to measure, it may be obvious that the ability to inhibit growth of or to kill possible pathogenic bacteria in the gastrointestinal tract will not only give this bacterium a competitive advantage within this environment but will also directly benefit the human host. Localized bacteriocin production is an important attribute of probiotic strains, enabling them to become established and to dominate their environment. In addition to the *abp118* structural genes, *Lactobacillus salivarius* UCC118 appears to contain genetic information encoding additional bacteriocin-like peptides, which are either not bioactive or not expressed/secreted, or which do not affect the indicator strains used in this study. Future investigations on the *abp118* locus are continuing and will, among other things, focus on the regulatory aspects of ABP-118 production.

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